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# Effects of Antioxidant Supplementation with Vitamin E and Selenium on Cytokine Production in Mice Infected with *Trypanosoma Cruzi*

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**EFFECTS OF ANTIOXIDANT SUPPLEMENTATION WITH VITAMIN E AND  
SELENIUM ON CYTOKINE PRODUCTION IN MICE INFECTED WITH  
TRYPANOSOMA CRUZI**

**A Thesis**

**Presented to the faculty of the Department of Biology  
Western Kentucky University  
Bowling Green, Kentucky**

**In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science**

**By**

**Dong Chen**

**December 2001**



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SELENIUM ON CYTOKINE PRODUCTION IN MICE INFECTED WITH  
*TRYPANOSOMA CRUZI***

Dong Chen

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64 Pages

Directed by: Cheryl D. Davis, Sigrid Jacobshagen, and Claire Rinehart

Department of Biology

Dietary supplementation with the antioxidants vitamin E and selenium (Se) has been shown to be beneficial against the intracellular parasite *Trypanosoma cruzi* during experimental Chagas' disease. Supplementation of mice with vitamin E and selenium at levels well above the recommended daily allowance for these two substances results in significantly decreased parasitemia levels and increased longevity. However, the mechanistic role of the antioxidants in this process is not well understood. It is known that vitamin E and selenium can reduce oxidative stress and improve host immune responses. In addition, the synthesis of many regulatory cytokines is known to be influenced by changes in the cellular oxidant/antioxidant balance. The present study was performed to determine the impact of the antioxidants vitamin E and selenium on the T helper (Th) 1/Th2 cytokine balance during experimental Chagas' disease. In the first phase of the study, two groups of female C3HeB/FeJ mice (24 mice each) were supplemented with vitamin E and selenium for 4-5 weeks and 8-9 weeks, respectively. In the second phase, 12 mice from each group were infected with a Brazil strain of *Trypanosoma cruzi*. In the final phase, levels of interferon gamma (IFN- $\gamma$ ) and interleukin 10 (IL-10) in serum were measured by antigen-capture enzyme linked immunosorbent

assay (ELISA). Transcriptional levels of IFN- $\gamma$  and IL-10 message RNA (mRNA) in the heart and spleen were measured by a ribonuclease protection assay. The results of the study confirmed the previously observed beneficial effect of antioxidant supplementation during murine infection with *T. cruzi*. In addition, supplementation of mice with vitamin E and selenium was shown to affect IFN- $\gamma$  and IL-10 synthesis at both the serum level and transcriptional level. Furthermore, IFN- $\gamma$  and IL-10 levels differed in the heart and spleen of infected mice. The Th1/Th2 cytokine balance was shown to be predominantly Th1 in mice receiving antioxidant supplementation. Vitamin E and selenium apparently exert a protective effect by enhancing IFN- $\gamma$  levels in mice infected with *T. cruzi* while decreasing the levels of IL-10 in non-infected, antioxidant-supplemented mice.



## BACKGROUND

### *Trypanosoma cruzi* and Chagas' disease

*Trypanosoma cruzi*, the causative agent of Chagas' disease, is transmitted by triatomine insects or by blood transfusion. *Trypanosoma cruzi* is a flagellated eukaryotic protozoan. The parasite is polymorphic and shows 3 different developmental stages in its life cycle: trypomastigote, amastigote and epimastigote.

The trypomastigote is the infective stage of *T. cruzi* and the only form transmitted between mammalian hosts and the invertebrate vector. It has a slender C-shape and is 12 to 20µm long and 2µm in width (Wendel and Gonzaga, 1993). The vectors of *T. cruzi* are hematophagous bugs of the family Reduviidae (Kissing bugs). *Panstrongylus*, *Triatoma* and *Rhodnius* are three genera of this family that are important in the spread of Chagas' disease to humans.

Within the midgut of the insect, trypomastigotes transform into short epimastigote stages, which multiply by longitudinal fission. The epimastigote is the replicative form of the parasite in the invertebrate host. After passing to the posterior intestine, they differentiate into the metacyclic trypomastigote stage. These infective trypomastigotes appear in the insect rectum 8 to 10 days after the blood meal (Roberts and Janovy, 1996). The bugs puncture the skin of the host in order to take a blood meal, and if the infective trypomastigotes are contained in feces, they gain entry into the host through the bite wound, scratched skin, or across mucous membranes that are rubbed with contaminated

fingers. After entry into the body of the mammalian host, trypomastigotes invade various nucleated cells. The most frequent cells are macrophages, muscle cells, nerve cells and reticuloendothelial cells of spleen, liver and lymphatics. Once in these cells, the trypomastigotes transform into the amastigote stage.

Amastigotes are spheroid, approximately 1.5 to 4.0  $\mu\text{m}$  wide, and lack a flagellum. They are the intracellular replicative forms of *T. cruzi* in mammalian cells. After reproducing several times, amastigotes transform back to the trypomastigote stage. Blood-form trypomastigotes (BFTs) are released from ruptured cells and then infect other host cells. When circulating trypomastigotes are present in peripheral blood, they can infect the insect vector during a blood meal.

### **Symptoms and pathogenesis**

There are three clinical stages in Chagas' disease: an acute stage, an indeterminant stage, and a chronic stage. The acute stage begins with the entrance of metacyclic trypomastigotes into the host's cells. Local inflammation at the wound site results in the formation of a small red nodule, known as a chagoma, with accompanying swelling of regional lymph nodes. Chagoma of the eye (Romana's sign) is seen frequently (Roberts and Janovy, 1996).

Acute myocarditis of different degrees also may occur. If parasites infect the nervous system, acute meningoencephalitis can develop and histopathological lesions in the meninges can be observed. Generally, the younger the patient, the more severe the clinical manifestations. Fatal diseases frequently occur in patients under two years old (WHO, 1991). In the acute stage, general malaise occurs along with a variety of possible clinical manifestation including the following: fever, enlarged liver and spleen,

generalized edema, swollen lymph nodes, sometimes a generalized exanthematous rash, anorexia, diarrhea, and vomiting.

The indeterminant stage is clinically asymptomatic and may last several years or persist indefinitely. In the chronic stage, patients suffer from cardiac, digestive, or neurological damage 10-20 years after being infected with parasite. The inflammatory process is most frequently focused in the heart, which results in chronic myocarditis and varying degrees of cardiac hypertrophy and dilation. If infection occurs within the esophagus and colon, mega-syndrome may result in the G. I. tract. This chronic pathology results from the continued presence of the parasite and a massive and progressive physiological loss of neurons, which are incapable of regeneration.

Patients with Chagasic heart disease develop severe cardiomyopathy leading to varying degrees of cardiac hypertrophy, dilation, and congestive heart failure that is caused by the destruction of parasympathetic ganglia, myocardium, and heart conduction fibers (WHO, 1991).

### **Host immunity to *T. cruzi***

Both natural resistance and acquired immunity are involved in the defensive response of the host following *T. cruzi* infection. Amphibians and birds are completely resistant to *T. cruzi*; the mammalian host cannot destroy infective trypomastigotes and allows them to remain alive for several weeks until parasite-specific antibodies are formed. Once the antibodies are formed, trypomastigotes may be killed by the activation of complement lysis. Cytotoxic T lymphocytes, macrophages, neutrophils, eosinophils and natural killer cells also have been shown to participate in immunological defense

during infection. The cell-mediated immune response against *T. cruzi* has been shown to be critically important, especially during the acute phase (Roberts & Janovy, 1996).

Interferon-gamma, CD8<sup>+</sup> T lymphocytes, macrophages, and tumor necrosis factor-beta (TNF- $\beta$ ) play important roles in controlling parasite replication. Within macrophages, nitric oxide synthesis is a major protective mechanism against intracellular parasitic infection (Green et al., 1991).

During the very early stages of experimental *T. cruzi* infection, immunosuppression occurs and contributes to the pathological effects. Production of the cytokine interleukin-2 (IL-2) and one subunit of the IL-2 receptor are suppressed early in infection, and the subsequent severe nonspecific immunosuppression that follows is a well-known hallmark of Chagas' disease. One consequence is that T cells can't be activated by antigen, mitogen, or anti-CD3 monoclonal antibody. However, levels of many other cytokines increase dramatically during infection, such as IFN- $\gamma$  and IL-10 (Zhang & Tarleton, 1996).

CD4<sup>+</sup> T lymphocytes are required in mediating host resistance during chronic Chagas' disease (Brener & Gazzinelli, 1997). Th1 cells play a helper/regulatory role in CD8<sup>+</sup> T cell cytotoxicity directed against *T. cruzi*-infected cells. In addition, parasite-specific Th1 cells activate macrophages to kill intracellular amastigotes of *T. cruzi* by a mechanism exclusively dependent on the induction of nitric oxide synthesis (Rodrigues et al., 2000).

Compared with cellular responses, antibody production is secondary but appears to be necessary for control of *T. cruzi* (Kumar & Tarleton, 1998). Humoral responses control chronic infections in which immunoglobulin M (IgM) and Immunoglobulin G

(IgG) play major roles. Both protective and non-protective antibodies can be found in hosts infected with *T. cruzi*. Protective antibodies, such as some circulate IgGs, function in opsonization, complement fixation and lysis of living trypomastigotes.

Autoantibodies have been hypothesized to play a role in the pathology of Chagas' disease for many years. However, more recent evidence suggests that the continued presence of *T. cruzi* in host tissues stimulates a chronic inflammatory response with infiltration of lymphocytes and macrophages (Tarleton & Zhang, 1999).

### **Cytokines and Th1/Th2 balance**

Cytokines are low molecular weight regulatory proteins or glycoproteins secreted by leucocytes and various other cells in the body in response to a number of stimuli. Their production is carefully regulated. They serve as chemical messengers of the immune system by binding to receptors present on numerous types of target cells. Their primary function is to regulate the proliferation, differentiation, and effector activity of immune-system cells. Many types of cells can secrete cytokines, such as T<sub>helper</sub> cells, B-lymphocytes, NK cells, macrophages, mast cells and other cell types. CD4<sup>+</sup> T<sub>helper</sub> (Th) lymphocytes exert most of their helper functions through the action of secreted cytokines. The differences in cytokine-secretion patterns among Th-cell subsets can determine the type of immune response made to a particular antigenic challenge. Two CD4<sup>+</sup> Th-cell subpopulations can be discriminated by the cytokine-patterns they secrete. These two subpopulations are designated as Th1 and Th2. Whereas Th1 cells are required for the development of immune control to *T. cruzi*, Th2 cells contribute to parasite persistence and increased severity of disease (Tarleton et al., 2000).

The Th1 subset has a cytokine profile consisting of high levels of IL-2, IFN- $\gamma$ , TNF- $\beta$  and GM-CSF that act primarily in cell-mediated responses. Th1 cytokines can promote macrophage function necessary to destroy internalized parasites. However, Th1 cytokines may also be responsible for the excessive inflammation and tissue injury. In a Th2-type immune response, there are high levels of IL-10, IL-4, IL-5 and IL-13, which can function in B-cell activation and in the stimulation of the humoral response while down-regulating macrophage activation. The Th2 subset is involved in the activation of eosinophils and participates in the type I hypersensitivity reaction. Th1 and Th2 regulatory cytokines stimulate the growth of the subset that produces them and cross-regulate the other subset in order to maintain the Th1/Th2 balance. In this way, cytokines can determine the progression and outcome of many diseases. The increased production of cytokines derived from the Th2 subpopulation of CD4<sup>+</sup> T cells may cause a fatal outcome in chronic human chagasic disease (Reis et al., 1997).

IFN- $\gamma$  is a defining cytokine of the Th1 subset and is produced by Th1 cells and NK cells. IFN- $\gamma$  can activate macrophages to increase microbicidal activity, it can increase NK cell activity, and it can up-regulate the level of class II MHC expression and the secretion of cytokines such as IL-12, which induce Th cells to differentiate into the Th1 subset (Boeham et al., 1997). IFN- $\gamma$  also promotes the production of certain isotypes of antibody. IFN- $\gamma$  and TNF- $\alpha$  are cytokines that mediate inflammation. Through their production, Th1 cells can impact inflammatory phenomena such as delayed-type hypersensitivity. Th1 cells also produce IL-2 and IFN- $\gamma$ , which promote the differentiation of fully cytotoxic T cells from CD8<sup>+</sup> precursors. This pattern of cytokine production makes the Th1 subset particularly well suited to respond to viral infection and

infection by other intracellular pathogens. In addition, IFN- $\gamma$  inhibits the expansion of the Th2 population.

IFN- $\gamma$  is crucial in the determination of resistance or susceptibility during infection with *T. cruzi*. In resistant animals, there is strong IL-18 gene expression in vivo, which results in an enhancement of INF- $\gamma$  production (Meyer et al., 1997). IL-18 is known to be an IL-12 and IFN- $\gamma$ -inducing cytokine and IFN- $\gamma$ -inducing factor. Nitric oxide (NO) is derived from macrophages and plays a critical role against different kinds of pathogens such as bacteria, viruses, fungi, and protozoan parasites. Nitric oxide has been shown to be a crucial killing effector molecule in resistance to intracellular stages of *T. cruzi* (Holscher et al., 1998). IFN- $\gamma$  is necessary for NO production that is mediated by the inducible nitric oxide synthase (NOS).

IL-10, secreted by Th2 cells, is an anti-inflammatory cytokine which participates in the cross-regulation of Th1 cells and has immunosuppressive functions through the down-regulation of IFN- $\gamma$  (D'Andrea et al., 1993). IL-10 indirectly inhibits Th1 cells by acting on monocytes, macrophages and other antigen presenting cells. IL-10 down-regulates the expression of class II MHC molecule on these antigen-presenting cells, interfering with their ability to activate the Th1 subset. IL-10 also acts on the monocyte-macrophage lineage to suppress the production of nitric oxide and other metabolites involved in the destruction of pathogens. The production of various inflammatory mediators (IL-11, IL-6, IL-8, GM-CSF,  $\alpha$ -CSF and TNF- $\alpha$ ) also is inhibited. Two Th2 cytokines, IL-10 and IL-4, down-regulate the secretion of IL-12, which is the critical cytokine of Th1 differentiation. The suppressive effects on macrophages by Th2 subset cytokines can further diminish the biological consequences of Th1 activation. The

function of T-cell subpopulations in immune regulation during *T. cruzi* infection is still not completely understood. However, cytokine control is very important during *T. cruzi* infection. Th1 cytokines have been shown to play a vital role in the control of parasite infections. In addition, there is a critical requirement for IL-10 to prevent the development of potential tissue pathology associated with CD4<sup>+</sup> T cells and the overproduction of IL-12 and IFN- $\gamma$  (Hunter et al., 1997).

During experimentally induced Chagas' disease, cytokines play key roles in the regulation of both parasite replication and the immune response in infected hosts. A Th1 immune response characterized by IFN- $\gamma$  production and subsequent activation of macrophages plays a major role in resistance to acute *T. cruzi* infection. IFN- $\gamma$  is particularly important in the very early stage of *T. cruzi* infection in determining disease outcome. By day 60 or later in infection, the balance of type 1 and type 2 cytokines is shifted in favor of IL-4 and IL-10 (Talvani et al., 2000). Endogenous IL-10 can down-regulate the pro-inflammatory cytokine response, which unchecked can lead to TNF- $\alpha$ -mediated toxic shock (Holscher et al., 2000). The balance between protective (e.g., IFN- $\gamma$ ) and exacerbative cytokines (e.g., IL-4 or IL-10) could be responsible for disease control and progression (Meyer et al., 1998). Although it is not a major determinant of susceptibility to Y strain *T. cruzi* infection, endogenous IL-4 along with IL-10, modulates IFN- $\gamma$  production and resistance (Abrahamsohn et al., 2000). Chronic human Chagas' disease is associated with increased systemic production of type 2 cytokines in response to *T. cruzi* infection and may be involved in the reciprocal down-regulation of IL-2 production (Samudio et al., 1998).



Neutralization of endogenous IL-10 in vivo increases resistance to infection with *T. cruzi* by increasing inflammatory cytokine responses, thus leading to reduced susceptibility (Dai et al., 1997). However, IL-10 prevents the development of a pathological immune response, which is correlated with CD4<sup>+</sup> T cells and the overproduction of IFN- $\gamma$ . Interleukin-10-deficient mice died within the third week of infection with *T. cruzi*, whereas all control mice survived acute infection (Holscher et al., 2000). In *T. cruzi*-infected IL-10<sup>-/-</sup> mice TNF- $\alpha$  was shown to be the direct mediator of mortality due to toxic shock. The dual role of IL-10 is illustrated by the observation that a reduced parasitemia but caused increased mortality occurs in IL-10 deficient mice infected with *T. cruzi* (Abrahamsohn & Coffman, 1996).

#### **Antioxidant nutrient and Immune system**

Single oxygen radicals, free radicals, and other reactive metabolites of oxygen are the by-products of metabolism. One family of molecules derived from the partial reduction of molecular oxygen is the reactive oxygen intermediates (ROI) that are released by the host from stimulated granulocytes and macrophages. Because of their free radical nature (i.e., the presence of an unpaired electron) and loss of spine restriction, ROI can react avidly with organic molecules and their active oxygen intermediates causing damage to DNA, proteins, and polyunsaturated membrane phospholipids in living organisms by oxidation.

The immune system can produce and use free radicals and reactive oxygen molecules, especially during the early stages of infection (Deshpande et al., 1996). The production of reactive oxygen intermediates is beneficial in the destruction of pathogens such as bacteria, viruses, fungi, and protozoan parasites. However, overproduction of free

radicals and reactive oxygen molecules can injure the immune cells themselves, as well as neighboring cells and tissues (Truyens et al., 1999).

Under normal conditions, free radicals are removed as soon as they are formed. For example, hydrogen peroxide is metabolized by the antioxidant enzyme, glutathione peroxidase (Diplock, 1991). Superoxide dismutase (SOD) can remove the superoxide anion radical in the mitochondrial compartment. The activity of these and other antioxidant enzymes is critical for preventing the accumulation of free radicals. SOD is copper and zinc dependent; therefore, its function is dependent on the presence of nutritional antioxidant minerals, such as copper, zinc, manganese, and selenium. Vitamin E, vitamin C, and  $\beta$ -carotene exert their antioxidant function by the formation of pre-oxidant substances instead of highly reactive free radicals. Pre-oxidants are much less reactive and have lower energy levels than the free radicals that they quench (Deshpande et al., 1996). Besides the capacity to scavenge free radicals, these antioxidant mechanisms are also important in disease prevention. For example, vitamin E plays an important role in the regulation of membrane fluidity and stabilization, 5-lipoxygenase activity, and protein kinase C activities (Blumberg, 1995).

Many studies also have shown that antioxidants can reduce the risk of some cancers, cardiovascular disease, infectious diseases, and damage due to pro-oxidant environmental pollutants such as smog and cigarette smoke. In addition, antioxidants can provide protection against inflammation and autoimmune disease (Deshpande et al., 1996). The antioxidant defense mechanism will be negatively affected when there is a deficiency in any of these nutrients.

It has been demonstrated that there is an increased production of reactive oxygen species by cells from mice acutely infected with *T. cruzi* (Cardoni et al, 1990). In addition, increase ROI production parallels the parasite burden during the acute phase of Chagas' disease.

Many experiments have shown that clinically relevant immune functions can be enhanced safely and effectively by supplementation with vitamin C, vitamin E, or  $\beta$ -carotene during conditions of increased oxidative stress (De la Fuente et al., 1998). Previous studies in our laboratory have provided evidence for a beneficial effect of elevated levels of vitamin E and selenium in the diet of mice infected with *T. cruzi*. The effects observed included reduced parasitemias, increased survival rates, and reduced weight loss during infection (Bennett, 1995; Hou, 1997).

### **Vitamin E**

Vitamin E, tocopherol, has been known as an antioxidant and immunostimulator for many years. As a lipid-soluble antioxidant, vitamin E is essential for the integrity and optimal functioning of all mammalian cells. This antioxidant property is the primary biological function of vitamin E. Vitamin E can stabilize biological membranes by protecting polyunsaturated fatty acids and other components of cell and organelle membranes from oxidation by reactive oxygen intermediates (Tappel 1972; Dutta-Roy et al., 1994). Cellular structures also can survive the damage of oxygen free radicals and reactive products of lipid peroxidation in the presence of vitamin E (Deshpande et al, 1996).

Vitamin E also has beneficial effects on immune function. It can protect the immune system from the harmful effects of free radical reactions and high levels of

vitamin E can have a stimulatory effect on the immune system. High doses of vitamin E in animals can stimulate cell-mediated immune function and increase humoral antibody production. The beneficial functions of vitamin E include the enhancement of T-cell mitogenesis, IL-2 production, T helper cell activity, B-cell mitogenesis, NK activity, macrophage phagocytosis, antibody titer, and DTH responses (Moriguchi and Muraga, 2000)

Deficiencies in vitamin E lead to a decline or suppression of the immune response. In addition, vitamin E deficiencies negatively affect both the maturation of specific lymphocyte subpopulations as well as the functional and proliferative capabilities of the peripheral lymphocytes (Chang et al., 1994).

Vitamin E is rich in dark green vegetables and vegetable oils, but generally poor in animal products (Budowski and Sklan, 1989). The most widely available form of vitamin E in food is  $\alpha$ -tocopherol which demonstrates the highest biological activity. International units (IU) are usually used to express the amount of vitamin E present. Two mg of  $\alpha$ -tocopherol equal to 3 IU (Dutta-roy, et al., 1994). The recommended dietary allowance (RDA) of vitamin E for men and women is 15 IU and 12 IU/person /day, respectively (Meydani, 1995).

### **Selenium**

Selenium is an antioxidant mineral and can be found in a variety of food sources, such as fish, meat, butter, cabbage, celery, radishes, garlic, and whole grains. Selenium is important to all mammalian cells because it is an essential component of several enzymes, which function in redox reactions. Spallholz (1994) reported that selenium is essential for the integrity of glutathione peroxidase (GSH-PX), an antioxidant enzyme

that can reduce intracellular peroxides and functions as a cellular protector against oxidative damage. Selenoprotein P is another major participant in selenium metabolism (Burk and Hill, 1994).

Selenium also is required for normal growth and development and functions as an antioxidant at the cellular level. Selenium has been shown to have anticarcinogenic function in vivo and in vitro and can decrease the risk of some cancers such as ovarian cancer (Stenson, 1996). Dietary intake of selenium affects immune function. Selenium supplementation increases the production of some classes of antibody, such as IgM and IgG (Spallholz et al, 1973) and significantly increases splenic lymphocyte activity, cytotoxic T lymphocyte-mediated tumor cytotoxicity, and lymphokine-activated killer cells. Macrophage and natural killer (NK) cell activity also has been shown to be enhanced during selenium supplementation compared to baseline values (Kiremidjian-Schumacher et al., 1994). Selenium also can make up for age-related deficiencies in lymphocyte proliferation and differentiation into cytotoxic effector cells. The molecular mechanisms involved in the impact of selenium on immune cell function may not be related to the function of selenium as an antioxidant or to gene activation (Kiremidjian-Schumacher and Roy, 1998).

Both deficiency and excess of selenium have the potential to be damaging to the immune system and other animal systems (WHO, 1987). Long-term selenium deficiency has been known to contribute to two diseases: (1) Keshan disease, which is a cardiomyopathy of children and young adults; (2) Kachin Bech diseases, which are osteoarthropathies of young people (WHO, 1987). Selenium supplementation can prevent both of these diseases.

The impact of selenium supplementation during bacterial, yeast, parasitic, and viral infection is somewhat variable. In some models of infectious disease selenium reportedly increases resistance, while in other systems it may actually impair the host response (Boyne et al., 1984).

### **Combined Effect of Vitamin E and Selenium**

Both vitamin E and Se function as antioxidants; either of them can impact the immune system and can provide protection against some diseases in humans and other animals. Vitamin E and selenium show a positive synergistic effect on the immune system when supplemented in quantities in excess of recommended levels (Scheffy and Schultz, 1979). Combined supplementation of Se and vitamin E result in enhanced macrophage function (Wise and Tomasso, 1993) and also enhanced antibody production, cell proliferation, cytokine production, prostaglandin metabolism, and neutrophil function (Desphande et al, 1996). Vitamin E and selenium also show a synergistic effect in depressing lipid peroxide formation. The combination of 400IU/kg vitamin E and 8ppm selenium provided the best overall protective effect in a murine model of experimental Chagas' disease (Hou, 1997). Douillet et al. (1996) showed that Vitamin E and Se exerted a synergistic beneficial effect in protecting kidney damage in diabetic rats. A significant correction of renal hyperfiltration and a diminution of the number and severity of glomerular lesions was observed in rats supplemented with both antioxidants.

Selenium and vitamin E showed overlapping and synergistic functions in an experiment in which C57B1/6J mice were fed different levels of these antioxidants during infection with coxsackievirus B3 (CVB3) (Williams et al., 1994). However, the two antioxidants have different functions and often can't compensate for each other.

Vitamin E protects lipid membranes against oxidative stress in two ways: by catalyzing the destruction of hydrogen peroxide or by catalyzing the decomposition of lipid hydroperoxides. Selenium acts as a component of glutathione peroxidase (GPX) in the cytoplasm and protects –SH groups in membrane proteins against oxidation (Zhu et al., 1992). Generally speaking, Se can prevent free radical production effectively, whereas vitamin E plays a role in preventing lipid peroxidation.

## INTRODUCTION

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), infects an estimated 18- 20 million people throughout South and Central America and frequently results in a chronic heart or digestive tract pathology known as Chagas' disease. In endemic areas, Chagas' disease remains the leading cause of heart disease and sudden death in young adults (WHO, 1991). Even though Chagas' disease and the etiologic agent, *T. cruzi*, have been well studied, there is currently no vaccine or effective treatment to control this infection. In general, supplementation with antioxidants such as vitamin E and selenium (Se) has been associated with an enhancement of the immune response and reportedly increases resistance to a variety of pathogenic infections and tumors in animal models and in humans (Wang et al., 1994; Meydani, 1995; Morrissey and Sheehy, 1999). Most recently, Han et al. (2000) demonstrated that vitamin E supplementation provides benefit in a murine model of influenza infection. Mice supplemented with vitamin E showed lower viral titers, and decreased weight loss following influenza infection. Likewise, previous studies in our laboratory have shown that supplementation of mice with Se (Davis et al., 1998) or vitamin E and Se (Hou, 1997) provides benefit during infection with the intracellular parasite, *Trypanosoma cruzi*. Dietary supplementation with vitamin E and Se in mice infected with *T. cruzi* resulted in increased longevity, decreased parasitemias, and reduced weight loss during infection.



Lymphocytes known as T helper (Th) cells (or CD 4+ T cells) are known to play an important role in the regulation and stimulation of immune responses. There are two currently recognized subsets of Th cells; type I (Th1) and type II (Th2). Th1 cells produce the cytokines interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) and promote cellular defense mechanisms. In contrast, Th2 cells produce the cytokines IL-4, 5, 6, 9, 10, and 13, which mediate humoral responses including allergic inflammation and asthma. The balance between Th1 and Th2 cytokines influences the consequence of infection by directing the immunological defense toward cell-mediated or antibody-mediated responses. Furthermore, there is reciprocal inhibition, in that Th1-type cytokines inhibit the production of Th2-type cytokines and vice versa.

The primary goal of this study was to investigate the impact of antioxidant supplementation on the cytokine response during *T. cruzi* infection. Cytokine profiles (especially IFN- $\gamma$  and IL-10) within the hearts, spleen, and sera of *T. cruzi*-infected mice were determined. Cytokine production was analyzed at the protein level by antigen capture enzyme-linked immunosorbent assay (ELISA) of mouse sera and culture supernatants from cultured murine spleen cells. Cytokine profiles within the heart and spleen were analyzed at the mRNA level using a multi-probe ribonuclease protection assay (RPA). The results of the study indicate that the antioxidants vitamin E and Se can increase IFN- $\gamma$  levels and decrease IL-10 level in serum during infection with *T. cruzi*, therefore, shifting the T-helper type response toward a Th1 cell-mediated response. A Th1-type response also was observed at the transcriptional level in the hearts of mice supplemented with vitamin E and selenium.

## MATERIALS AND METHODS

### Mice and diets

Female syngeneic mice, strain C3HeB/FeJ (Jackson Laboratory, Bar Harbor, ME) were used in this study. Beginning at 5-6 weeks of age, mice were randomly separated into 4 cages of 6 mice each and were maintained at room temperature (26°C). Mice were immediately placed on one of the diets described below. Three cages of mice were given a specially formulated chow containing 400 IU/kg of vitamin E (Purina Mills, Richmond, IN). Distilled water-containing 8ppm sodium selenate was provided as drinking water. Mice in the remaining three cages were fed a common Purina rodent chow, #5001, which contained the recommended daily allowance of 0.2ppm Se and 40 IU/Kg vitamins E. Distilled H<sub>2</sub>O was provided as drinking water.

Two separate experiments were performed in which mice were treated for differing lengths of time prior to infection with *T. cruzi*. Food and water were provided *ad libitum* in both experiments.

In experiment #1, the groups were as follows (8-9 weeks of antioxidant treatment prior to infection):

Group A, 400IU/kg vitamin E plus 8 p.p.m. sodium selenate; assayed 35 days post-infection.

Group B, 400IU/kg vitamin E plus 8 p.p.m. sodium selenate; not infected.

Group C, (control) Standard Purina Rodent Chow, #5001, plus diH<sub>2</sub>O. Assayed 35 days post-infection.

Group D, (control) common Purina Rodent Chow, # 5001, plus diH<sub>2</sub>O, not infected.

In experiment #2, the groups used were as follows (4-5 weeks of antioxidant treatment prior to infection):

Group A, 400IU/kg vitamin E plus 8 p.p.m. sodium selenate; assay 37 days post-infection.

Group B, 400IU/kg vitamin E plus 8 p.p.m. sodium selenate; not infected.

Group C, (control) Standard Purina Rodent Chow, #5001, plus diH<sub>2</sub>O; assay 37 days post-infection.

Group D, (control) common Purina Rodent Chow, # 5001, plus diH<sub>2</sub>O, not infected.

All mice were maintained and used in accordance with NIH and local IACUC guidelines.

### **Parasites**

A Brazil strain of *Trypanosoma cruzi*, which is maintained in the laboratory as a stock infection in C3HeB/FeJ mice, was used for all experiments in this study. An infection inoculum of  $1 \times 10^3$  BFTs in 0.2 ml of Dulbecco's Phosphate Buffered Saline (DPBS, Sigma Chemical Co., St. Louis, Missouri) was administered intraperitoneally (I.P.) to each mouse.

### **Parasitemia**

Parasitemia levels were monitored in all infected mice at 3~4 days intervals starting at day 13 post-infection and continuing throughout the course of infection until mice were euthanized. Blood was obtained from each mouse by clipping the tip of the tail and squeezing the tail until a uniform round drop of blood was expelled. A 5 $\mu$ l sample of

blood was obtained from the tail of each mouse and was diluted in 95  $\mu$ l DPBS (making a 1/20 dilution). A well-mixed sample was counted using a hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania). Mice also were monitored daily to determine levels of mortality and morbidity.

### **Preparation of Serum**

Mice were anesthetized in a jar containing ether-soaked cotton. A 1cc syringe attached a 26 gauge needle was used to perform a cardiac puncture on the mouse drawing blood into the syringe. Blood was expelled into a small micro-centrifuge tube and was allowed to clot at RT for 1 hour and at 4°C overnight. The tube was centrifuged at a setting of 800 x g for 10 minutes. The supernatants were collected and stored at -80 °C until use.

### **Preparation of Spleen Cells**

The following procedures were performed under sterile conditions. Following the removal of blood by cardiac puncture, mice were killed by cervical dislocation. The abdomen of each mouse was doused with 70% ethanol. A cut was made through the loose skin in the inguinal region, and the skin was pulled back until the peritoneum was widely exposed. The loose hair was removed by flooding the peritoneal membrane with 70% ethanol. The peritoneal membrane was lifted over the spleen with forceps and a large U-shaped cut was made around the spleen. The peritoneum was folded back, and the spleen was lifted out with forceps. Connective tissue was separated from the spleen with scissors. The spleen was placed into a small petri dish containing RPMI-1640 medium. Spleen cells were released from the capsule by squeezing with large forceps. Once the spleen cells were dislodged, cells were drawn up into a 5cc syringe with a 22-gauge

needle and cells were expelled slowly into a 15cc tube through a 26-gauge needle in order to obtain a single-cell suspension. Erythrocytes were then lysed by hypotonic shock and cell counts were performed using a hemacytometer. Total spleen cells ( $4 \times 10^6$ ) were cultured in 1ml RPMI complete medium (RPMI 1640 medium plus 10% newborn calf serum, 25mM N-2 -hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES),  $5 \times 10^{-5}$  M-2-mercaptoethanol, 100u/ml penicillin G, 100mM streptomycin, 1mM glutamine, and 1mM pyruvate. Concanavalin A (ConA: 5ug/ml) was added to some of the spleen cell cultures to stimulate these cells to produce more cytokines. Culture supernatants were recovered at 48h and stored at  $-80^{\circ}\text{C}$  until enzyme-linked immune sorbant assay (ELISA) could be performed.

### **Cytokine ELISA**

For enzyme-linked immunosorbent assays (ELISA), 96-microwell plates were coated with capture antibodies (anti-mouse IL-10 or anti-mouse IFN- $\gamma$ ) and incubated over night. The incubation temperature for the IL-10 ELISA was  $37^{\circ}\text{C}$ . Incubations for the IFN- $\gamma$  assay were performed at RT. The plates were blocked for 1 hour with 1% bovine serum albumin (BSA) in blocking buffer. For the IFN- $\gamma$  assay, 5% sucrose in DPBS with 0.05%  $\text{NaN}_3$  was used as a blocking buffer. For the IL-10 assay, 0.25% Tween 20 in DPBS [Dulbecco's phosphate buffered saline] was used as a blocking buffer. Serial dilutions of purified recombinant IFN- $\gamma$  and IL-10 were used to generate standard curves and were incubated in parallel with serum samples and spleen cell culture supernatants for 2 hours. For the IL-10 assay, samples were first diluted 1:2 in sample diluent [PBS with 1% BSA 0.25% Tween 20 and 25% heat inactivated bovine calf serum]. Biotinylated anti-mouse IL-10 / IFN- $\gamma$  antibodies were incubated 1 hr or 2 hrs,

respectively. The plates were washed at 3 times with PBS-0.1% Tween 20. The substrate, 3,3', 5,5' tetramethylbenzidine (TMB; Sigma Chemical Co.), was added to each well and incubated at RT for ~30 min. An enzymatic color reaction was obtained. The optical densities were measured at a wavelength of 450nm with a microplate reader (BioRad). Duplicate readings for each standard and sample were averaged, and data were analyzed according to the protocol described by the manufacturer of the DuoSet ELISA Development System (R&D Systems, Inc., MN.).

### **RNA Isolation**

Mice were anesthetized by ether inhalation and blood was drawn for serological analysis by cardiac puncture as described above. Immediately following cervical dislocation, hearts and spleens were aseptically removed from each mouse, placed into sterile cryovials, and flash frozen in liquid nitrogen. The cryovials containing frozen spleen and heart tissue of both infected and control mice were stored at  $-80^{\circ}\text{C}$ . Total RNA was isolated from the heart and spleen cells of each mouse using TRIzol Reagent (Life Technologies GIBCOBRL, Gaithersburg, MD) according to the protocol suggested by the manufacturer. Sample tissue was homogenized in TRIzol-Reagent using a glass homogenizer with a Teflon-pestle. Phenol-chloroform was used for phase separation and samples were spun in a centrifuge at  $12,000\times g$  for 15 minutes at 2 to  $8^{\circ}\text{C}$ . The upper aqueous phase was collected from each sample and precipitated with 100% isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization). Then, samples were mixed by vortexing and were centrifuged no more than  $7,500\times g$  for 5 minutes at 2 to  $8^{\circ}\text{C}$ . The resulting RNA samples were re-dissolved in RNase-free water and were stored at  $-70^{\circ}\text{C}$  until use. RNA yield was quantified by

spectrophotometry, and RNA purity was confirmed by agarose gel electrophoresis. Following isolation, RNA samples were diluted in diethylpyrocarbonate-treated (DEPC) treated distilled water and stored frozen at -80°C until use.

### **Ribonuclease Protection Assay of Cytokine Gene Transcripts**

A Ribonuclease Protection Assay (RPA) was performed on RNA samples isolated from mouse hearts and spleens using the RiboQuant: Multi-probe RNase Protection Assay (RPA) System (BD Pharmingen) according to the manufacturer's protocol. The mCK-1 Mouse Cytokine Multi-Probe Template Set (Cat. #45001P, BD Pharmingen) was used in conjunction with the RiboQuant kit for analysis of Th1 and Th2 immunoregulatory cytokines. This template set contains DNA templates which were used for the T7 RNA polymerase-directed synthesis of high-specific activity [ $\alpha$ - $^{32}$ P]-labeled antisense RNA probes that were hybridized with target mouse mRNAs encoding IL-4, L-5, IL-10, L-13, IL-15, IL-9, IL-2, IL-6 and IFN- $\gamma$ , as well as two house keeping gene products: L32 [ribosomal protein L32] and GAPDH [glyceraldehyde-3-phosphate dehydrogenase]. Details of the probe synthesis are described below. Following hybridization and ribonuclease treatment, samples were electrophoresed on a denaturing 5% acrylamide sequencing gel. Diluted, unprotected radiolabeled probes were used as size markers. After electrophoresis, gels were dried under vacuum for 1 hour at 80°C. A sheet of Kodak X-AR film was placed over the dried gel and the entire assembly was placed into a cassette with an intensifying screen. Films were exposed at -80°C for 24~48 hours. Films were put in development solution for 30 seconds, fix solution 45 seconds (keep shaking), and H<sub>2</sub>O for rinsing 15 minutes. A standard curve was established using markers (undigested probes) and nucleotide lengths of experimental bands were

estimated. Autoradiographic band intensities were determined following scanning using Adobe Photoshop in conjunction with Kodak Digital Science™ computer software.

### **Probe synthesis**

A mCK-1 RPA Template set was used to synthesize probes that were labeled by [ $\alpha$ - $^{32}$ P] UTP (Amersham). RNasin, a GACU pool, DTT, 5X transcription buffer, the mCK-1 RPA Template Set, [ $\alpha$ - $^{32}$ P] UTP, and T7 RNA polymerase were placed in order into 1.5 ml microcentrifuge tubes. The samples were mixed well, subjected to a quick spin in a micro-centrifuge, and then incubated at 37°C for 1 hour. DNase was added to terminate the reaction, and samples were incubated at 37°C for 30 min. Twenty-six  $\mu$ l 20mM EDTA, 25 $\mu$ l Tris-saturated phenol [pH8.0], 25 $\mu$ l chloroform: isoamyl alcohol (50:1) and 2 $\mu$ l yeast tRNA (PharMingen) were then added, and the tube was mixed by vortexing for 5 min at room temperature. The upper aqueous phase was transferred to a new 1.5ml micro-centrifuge tube and 50  $\mu$ l chloroform: isoamyl alcohol (50:1) was added. The tubes were mixed by vortexing and subjected to a quick spin in a micro-centrifuge at room temp. The upper aqueous phase was transferred to a new 1.5ml micro-centrifuge tube. Fifty  $\mu$ l 4 M ammonium acetate and 250  $\mu$ l ice-cold 100% ethanol were added and the tubes were inverted, mixed, and incubated for 30 min at -70°C. Then the tubes were spun in a micro-centrifuge no more than 12,000x g for 15min at 4°C. The supernatant was removed, and 100  $\mu$ l ice-cold 90% ethanol was added to the pellet. After a five minute spin no more than 7,500 x g in a micro-centrifuge at 4°C, all of the supernatants were carefully removed and the pellet were air-dried for 5 to 10 min. Fifty  $\mu$ l of hybridization buffer [80% deionized formamide + 100 mM sodium citrate pH 6.4 + 300 mM sodium acetate pH 6.4 +1mM EDTA] was added and the pellets were



solubilized by gently vortexing for 20 sec. One  $\mu\text{l}$  sample duplicates were quantitated in the scintillation counter. A yield of  $\sim 3 \times 10^5$  to  $\sim 3 \times 10^6$  Cherenkov counts/ $\mu\text{l}$  was expected. The mRNAs were stored at  $-20^\circ\text{C}$ . The probes labeled with  $[\alpha\text{-}^{32}\text{P}]$  UTP were used for two successive overnight hybridizations.

### **RNA Preparation & Hybridization**

Twenty  $\mu\text{l}$  of target RNase (including a yeast tRNA as a background control and PharMingen control RNA) were placed into separate 1.5 ml micro-centrifuge tubes. Samples were dried completely ( $\sim 1$  hour) in a vacuum evaporator centrifuge (no heat). Eight  $\mu\text{l}$  of hybridization buffer was added to each sample and the RNA was solubilized by gentle vortexing for 3-4 min followed by a quick spin in a micro-centrifuge. Probes were diluted to  $2.9 \times 10^5$  Cherenkov counts / $\mu\text{l}$  and  $2 \mu\text{l}$  of diluted probe was added to each tube and quick spun in the microfuge. The oil added samples were placed into a heat block pre-warmed to  $90^\circ\text{C}$ . The temperature control knob was immediately turned to  $56^\circ\text{C}$  (allowing the temperature to ramp down slowly) and tubes were incubated for 12-16 hr. The heat block was set to  $37^\circ\text{C}$  for 15min prior to the RNase treatments.

### **RNase Treatments**

RNA samples were removed from the heat block and  $100 \mu\text{l}$  of the RNase cocktail [2.5ml RNase buffer plus  $6 \mu\text{l}$  RNase A + T1 mix per 20 samples] was pipeted underneath the oil (as a bubble) into the aqueous layer. Samples were spun in the micro-centrifuge for 10 sec and then incubated for 45 min at  $30^\circ\text{C}$ . The RNase digests were carefully extracted from underneath the oil and transferred to new micro-centrifuge tubes containing  $18 \mu\text{l}$  mixed proteinase K cocktail solution [ $390 \mu\text{l}$  Proteinase K buffer +  $30 \mu\text{l}$  Proteinase K +  $30 \mu\text{l}$  yeast tRNA per 20 samples]. The samples were then quickly

vortexed, spun in the microfuge, and incubated for 15 min at 37°C. Sixty-five µl Tris-saturated phenol and 65µl chloroform: isoamyl alcohol (50:1) were added to the tubes. The resulting suspension was then vortexed into an emulsion and spun in a micro-centrifuge no more than 12,000 x g for 5 min at RT. The upper aqueous phase was carefully extracted and transferred to a new tube. Ice-cold 100% ethanol and 120µl 4 M-ammonium acetate were added and tubes were inverted to mix the sample. Following 30 min incubation at -70°C, samples were spun in the micro-centrifuge no more than 12,000 for 15 min at 4°C. The supernatant was carefully removed and the pellet was air dried completely. A 5 µl aliquot of 1X loading buffer [95% formamide + 0.025% xylene cyanol + 0.025% bromophenol blue + 18 mM EDTA +0.025% SDS] was added, and the tubes were vortexed for 2-3 min. Following a quick spin in the micro-centrifuge, the samples were heated for 3 min at 90°C and placed immediately into an ice bath.

### **Gel Resolution of Protected Probes**

A set of gel plates (>40 cm in length) was thoroughly cleaned with water followed by ethanol. The short plate was siliconized and cleaned again. The gel mold was assembled using 0.4 mm spacers. A 5% acrylamide solution [74.5 ml acrylamide solution of a final 19:1 acrylamide/bis: 8.85 ml of 40% acrylamide + 9.31 ml of 2% bis acrylamide + 7.45 ml of 10x TBE + 35.82 g of Urea + QS to 74.5 ml with dH<sub>2</sub>O + 450 µl 10% ammonium persulfate + 60 µl TEMED] was prepared and poured immediately into the gel mold [10 x TBE: 108g Tris Base+ 55g Boric Acid + 40ml 0.5M EDTA, pH 8.0 QS to 1 liter dH<sub>2</sub>O]. Combs were added and the gel was allowed to polymerize for ~1 hour. The combs were removed and the wells were flushed thoroughly with 0.5x TBE [1 x TBE: 89mM Tris Base + 89mM Boric Acid + 2mM EDTA]. Gels were pre run at 40 watts

constant power for ~45 min, with 0.5 x TBE as the running buffer. Gel temperature was held constant at 50°C. The wells were flushed again with 0.5x TBE and samples were loaded. Diluted probes (1000-2000 Cherenkov /lane) were used as size markers. The gel was run at 50 watts constant power until the leading edge of the bromophenol blue (BPB) reached a distance of 30cm. The gel mold was disassembled, the short plate was removed, and the gel was adsorbed to filter paper. The gel was covered with Saran wrap and layered between two additional pieces of filter paper. The entire assembly was placed in the gel dryer under vacuum for ~1 hr at 80°C. The dried gel was placed on a sheet of X-ray film [Kodak X-AR] in a cassette with an intensifying screen and was developed at -70°C for 24 to 48 hr. With the undigested probes as markers, a standard curve of migration distance versus log nucleotide length was plotted on semi-log graph paper. This curve was used to establish the identity of “RNase-protected” bands in the experimental samples. After scanning the film Adobe Photoshop was used with the Kodak Digital Science <sup>TM</sup> 1D to quantify band intensities.

### **Statistical Evaluation**

Data were analyzed using SYSTAT 1999. ANOVA (Analysis of Variance) was used to test the effect of diet on mice infected with *T. cruzi*. Dependent variables in the ANOVA include the concentration of cytokines, the numbers of parasites. Weight of total RNA and the intensity of RNA band were given as the mean values per group of mice.

## RESULTS

### **Effect of vitamin E + Se supplementation on parasitemia**

Mean parasitemia levels for each treatment group in experiment 1 are shown in Figure 1. The mean parasitemia levels for mice in the vitamin E + Se treated group (group A) were lower ( $p < 0.05$ ) than the parasitemia levels of mice maintained on diets lacking in vitamin E and Se (group C) throughout the course of infection.

### **Effect of vitamin E + Se supplementation on IFN- $\gamma$ levels**

The mean IFN- $\gamma$  concentrations in serum obtained from mice in each group are shown in Figure 2. Vitamin E + Se-treated mice infected with *T. cruzi* (group A) showed higher IFN- $\gamma$  levels in serum than vitamin E + Se deficient *T. cruzi* infected mice (group C). Both older mice in experiment 1 and younger mice in experiment 2 showed statistically significant differences ( $F_1=6.71$ ,  $df_1=10$ ,  $p_1=0.027$ ;  $F_2=6.608$ ,  $df_2=10$ ,  $p_2=0.028$ ). Interferon gamma levels were not significantly different between the two groups of non-infected mice (groups B and D) in either experiment.

Mean IFN- $\gamma$  concentrations in Con-A-stimulated spleen cell cultures obtained from mice in each group are shown in Figure 3. Because levels of IFN- $\gamma$  in spleen cell cultures were very low and standard deviations were large, no statistically significant

differences were observed between the two groups of *T. cruzi*-infected mice in either experiment 1 or experiment 2.

### **Effect of vitamin E +Se supplementation on IL-10 levels**

Mean IL-10 concentrations in sera obtained from mice in each group are shown in Figure 4. Vitamin E and Se-treated but non-infected mice (group B) showed lower IL-10 levels as compared to antioxidant-deficient control mice (group D). This difference was statistically significant in both experiment 1 and in experiment 2 ( $F_1 = 6.032$ ,  $df_1 = 10$ ,  $p_1 = 0.034$ ;  $F_2 = 12.61$ ,  $df_2 = 10$ ,  $p_2 = 0.005$ ). However, *T. cruzi*-infected mice showed no statistically significant differences in serum IL-10 levels, regardless of diet.

IL-10 levels in ConA-stimulated spleen cell cultures are shown in Figure 5. Only low levels of IL-10 were detected in spleen cell cultures and standard deviations were very large. Only mice in group A in experiment 1 (vitamin E + Se and *T. cruzi*-infected) showed significantly higher levels of IL-10 as compared with *T. cruzi*-infected mice from group C (diet deficient in Vitamin E and Se;  $F = 6.187$ ,  $df = 10$ ,  $p = 0.032$ ). There were no statistically significant differences in IL-10 levels between other groups of mice.

### **Effect of vitamin E+ Se supplementation on total RNA production**

Mean values for total RNA isolated per spleen and heart tissue are shown in Figure 6. Although there were no statistically significant differences based upon levels of antioxidant supplementation, mice maintained on elevated levels of vitamin E + Se showed an obvious trend toward lower total RNA levels in both spleen and heart tissue. There were, however, statistically significant differences observed in total RNA levels between *T. cruzi*-infected and non-infected groups of mice in both spleen and heart tissue (spleen:  $F = 44.617$ ,  $df = 4$ ,  $p = 0$ ; heart:  $F = 6.534$ ,  $df = 4$ ,  $p = 0.029$ ). During infection with *T.*

*cruzi*, splenomegaly is very common in the mammalian host; infections represent a strong antigenic stimulation to the host. The total mass of the organs and the presence of infection affected the RNA production.

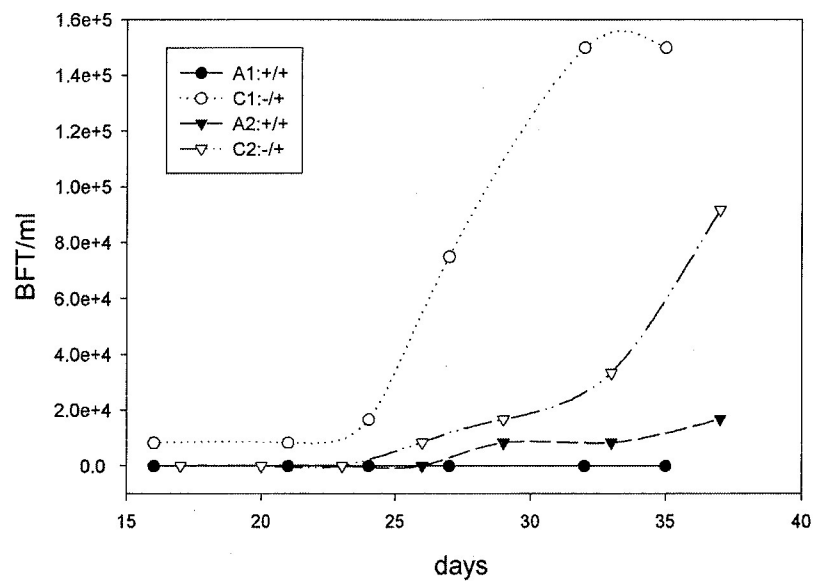
#### **Effect of diet on cytokine mRNA production in *T. cruzi*-infected C3HeB/FeJ mice**

According to the estimated intensity of cytokine mRNA bands which were normalized by the intensity of a house-keeping gene, expression of the following cytokines were increased in *T. cruzi*-infected mice: IFN- $\gamma$ , IL-10, IL-9 and IL-15 in both spleen and heart; IL-6 in heart; IL-4 in spleen (group B versus D in Table 1a+1b).

Antioxidant-treated mice showed an increase in the expression of the following cytokines as compared with control, non-supplemented mice: IFN- $\gamma$  in heart, IL-10 in spleen, IL-15 in heart, IL-6 in heart and IL-9 in both organs (group C versus D in Tables 1a + 1b). Antioxidant supplementation resulted in a decrease in the expression of the following cytokines: IFN- $\gamma$  in spleen, IL-10 in heart, and IL-15 in spleen. IL-5, IL-13, IL-12 levels in the heart and IL-6 levels in the spleen showed no change with response to infection or antioxidant supplementation.

**Figure 1. Effect of vitamin E and Se supplementation on parasitemia in *T. cruzi*-infected mice.** Points represent the mean number of blood-form trypomastigotes per milliliter of blood (6 mice per group; standard deviations are not indicated to preserve the clarity of the figure). “+/+”= Group A: vitamin E & Se (+), *T. cruzi* infection (+). “-/+” = Group C: vitamin E & Se (-), *T. cruzi* infection (+). The number 1 represents experiment 1 and the number 2 represents experiment 2.

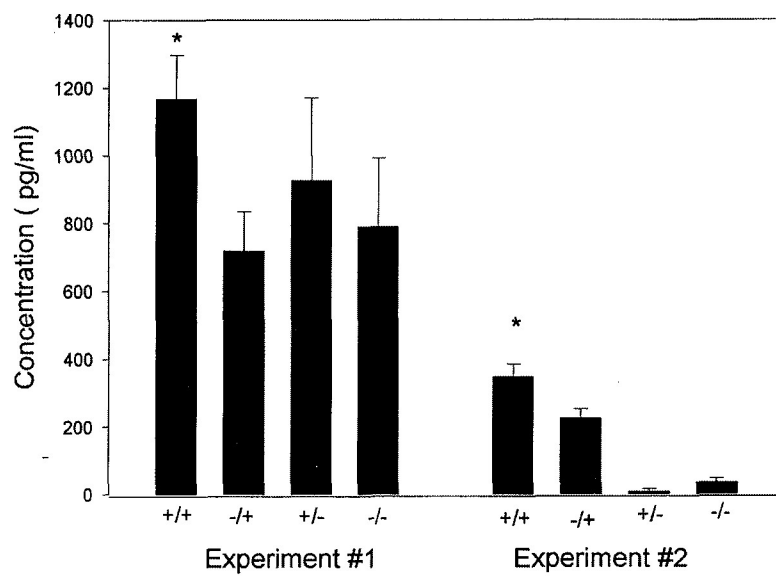
## Parasitemia





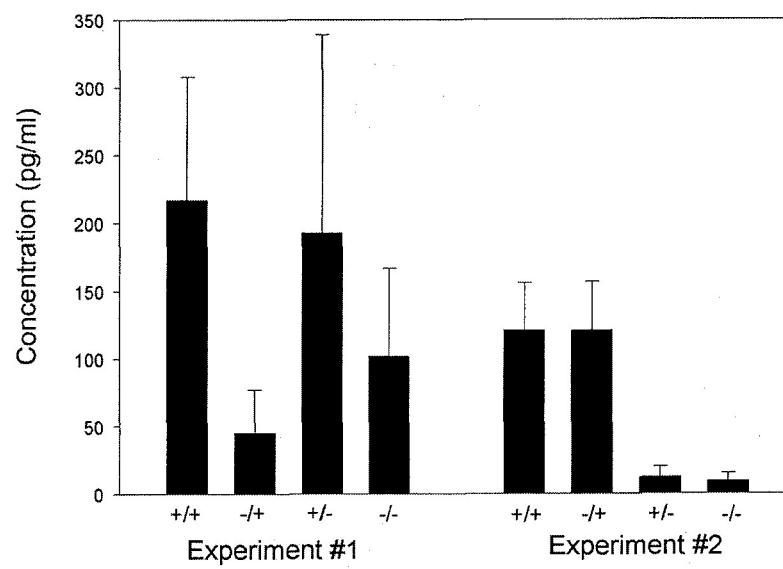
**Figure 2. Effect of vitamin E and Se supplementation on IFN-gamma levels in the serum of *T. cruzi*-infected mice.** The concentration of IFN- $\gamma$  present in the serum of mice in each group was measured on day 35 (experiment 1) and day 37 (experiment 2) post-infection, respectively. Data are presented as mean concentration plus standard deviation. Mice in experiment 1 were treated with vitamin E & Se for 72 days and were 15~16 weeks old when infected with *T. cruzi*; Mice in experiment 2 were treated with vitamin E and Se for 30 days and were 8~10 weeks old when infected with *T. cruzi*. “+/+” = Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-” = Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+” = Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-” = Group D: vitamin E & Se (-), *T. cruzi* infection (-). \* indicate statistically significant differences.

### Interferon-gamma Level in Serum



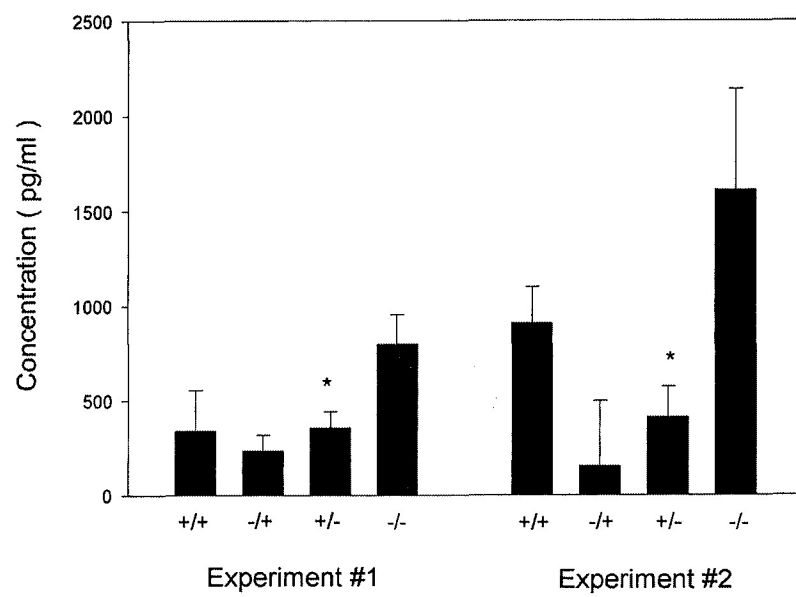
**Figure 3. Effect of vitamin E and Se supplementation on IFN- $\gamma$  levels in spleen cell cultures obtained from *T. cruzi*-infected mice.** The concentration of IFN- $\gamma$  released into culture supernatants for each group of mice was measured on day 35 post-infection + 48 hour of culture (experiment 1) and on day 37 post-infection+ 48 hour of culture (experiment 2) respectively. Data are presented as mean concentration plus standard deviation. In experiment 1, mice were treated with vitamin E & Se for 72 days and were 15~16 weeks old when infected with *T. cruzi*. In experiment 2, mice were treated with vitamin E and Se for 30 days and were 8~10 weeks old when infected with *T. cruzi*. “+/+”= Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-”= Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+”= Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-”= Group D: vitamin E & Se (-), *T. cruzi* infection (-). \* indicate statistically significant differences.

### Interferon-gamma Level in Spleen Cell Culture



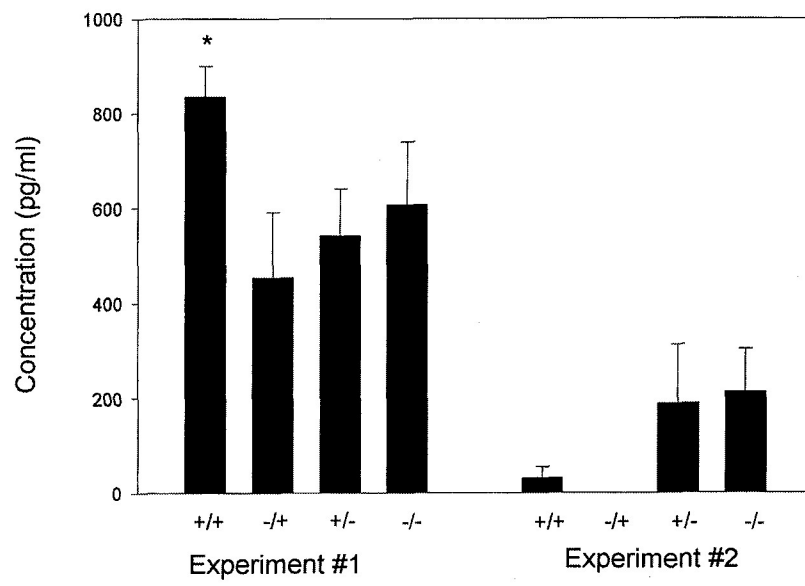
**Figure 4. Effect of vitamin E and Se supplementation on IL-10 levels in the serum of *T. cruzi* -infected mice.** The average concentration of IL-10 present in sera obtained from mice in each group was measured on day 35 (experiment 1) and day 37 (experiment 2) post-infection, respectively. Data are presented as average concentration plus standard deviation. Mice from experiment 1 were treated with vitamin E & Se for 72 days and were 15~16 weeks old when infected with *T. cruzi*. Mice from experiment 2 were treated with vitamin E and Se for 30 days and were 8~10 weeks old when infected with *T. cruzi*. “+/+”= Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-”= Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+”= Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-”= Group D: vitamin E & Se (-), *T. cruzi* infection (-). \* indicate statistically significant differences.

### IL-10 Level in Serum



**Figure 5. Effect of vitamin E and Se supplementation on IL-10 levels in spleen cultures obtained from *T. cruzi*- infected mice.** The concentration of IL-10 released into culture supernatants for each group of mice was measured on day 35 + 48 hours of culture (experiment 1) and day 37 + 48 hours of culture (experiment 2) post-infection, respectively. Data are presented as average concentration plus standard deviation. Mice from experiment 1 were treated with vitamin E & Se for 72 days and were 15~16 weeks old when infected with *T. cruzi*. Mice from experiment 2 were treated with vitamin E and Se for 30 days and were 8~10 weeks old when infected with *T. cruzi*. “+/+”= Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-”= Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+”= Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-”= Group D: vitamin E & Se (-), *T. cruzi* infection (-). \* indicate statistically significant differences.

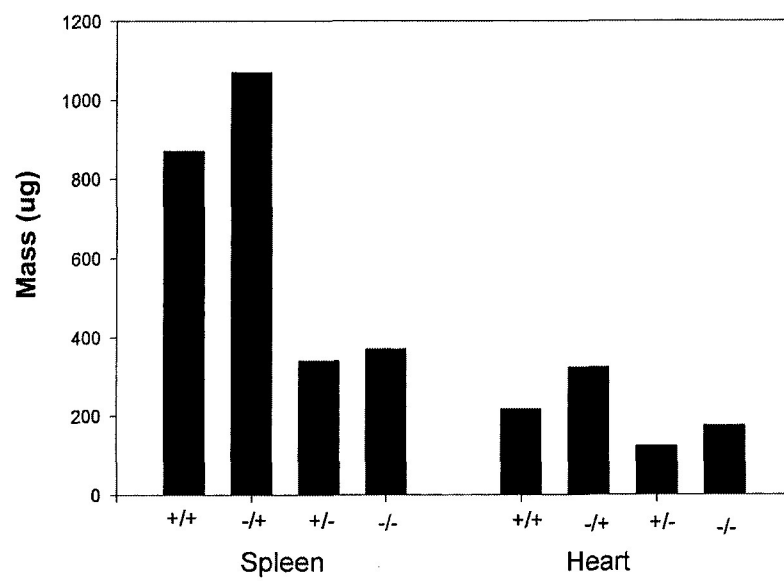
### IL-10 Level in Spleen Cell Culture





**Figure 6. Effect of vitamin E supplementation on total RNA levels** This assay was performed with mice from experiment 2# on day 37 post-infection. Data are presented as average RNA mass. “+/+”= Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-” = Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+”= Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-”= Group D: vitamin E & Se (-), *T. cruzi* infection (-).\* indicate statistically significant differences.

**Total RNA from Spleen and Heart Tissue**



**Figure 7. Effect of antioxidant supplementation on cytokine mRNA production in *T. cruzi*-infected mice.** One representative autoradiograph is shown here. RNA isolated from individual mice from each group in experiment 2 was analyzed by ribonuclease protection assay (RPA). The bands represent different cytokine mRNAs. Lanes 2~8 represent RNA isolated from spleen tissue; Lanes 9~16 represent RNAs isolated from heart tissue. “+/+” = Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-” = Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+” = Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-” = Group D: vitamin E & Se (-), *T. cruzi* infection (-). “1” and “2” mean individual mice from the same group.

## RPA Result

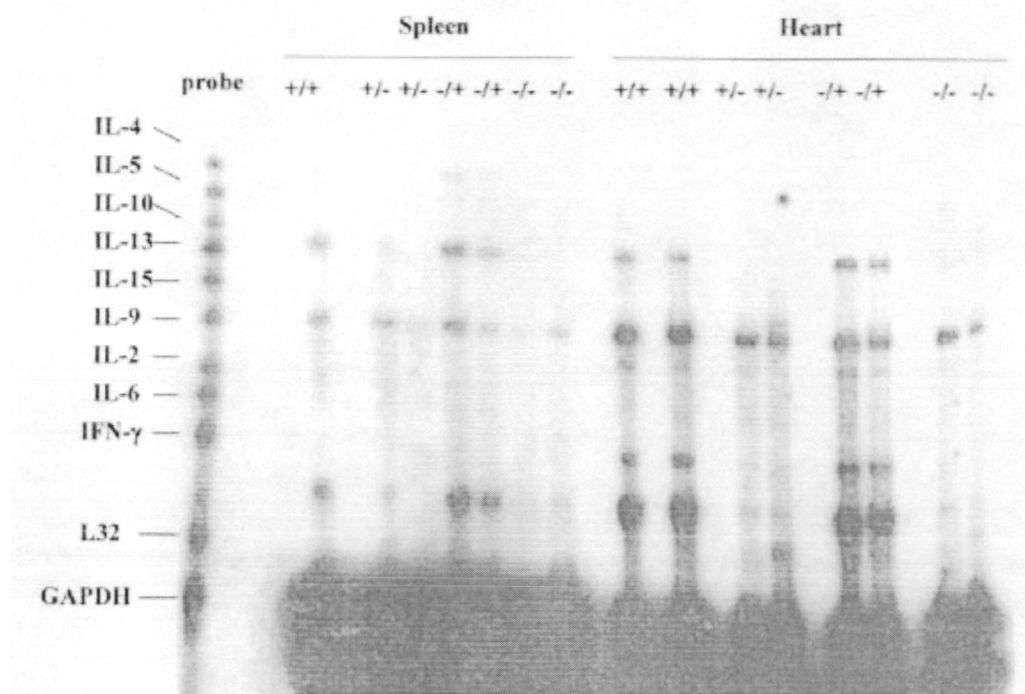


Table 1. a. Average intensity of cytokine mRNA bands from spleen tissue of individual mice in different treatment groups. “+/+” = Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-” = Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+” = Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-” = Group D: vitamin E & Se (-), *T. cruzi* infection (-).

b. Average intensity of cytokine mRNA bands from heart tissue of individual mice in different treatment groups. “+/+” = Group A: vitamin E & Se (+), *T. cruzi* infection (+). “+/-” = Group B: vitamin E & Se (+), *T. cruzi* infection (-). “-/+” = Group C: vitamin E & Se (-), *T. cruzi* infection (+). “-/-” = Group D: vitamin E & Se (-), *T. cruzi* infection (-).

Note: some band intensities indicated by a “0” were detectable, but after normalization the values were not significantly different than zero.

**Table 1**

a. Average intensity of cytokine-bands from spleen mRNA

Group	IL-4	IL-5	IL-10	IL-13	IL-15	IL-9	IL-2	IL-6	IFN-r
A (s)	22	0	99	0	100	62	0	0	122
B (s)	0	0	16	0	76	27	0	0	63
C (s)	35	0	85	0	104	40	42	0	165
D (s)	0	0	0	0	85	0	0	0	76

b. Average intensity of cytokine-bands from heart mRNA

Group	IL-4	IL-5	IL-10	IL-13	IL-15	IL-9	IL-2	IL-6	IFN-r
A (h)	0	0	91	0	167	124	0	152	220
B (h)	0	0	0	0	153	44	0	87	100
C (h)	29	0	115	0	156	120	0	151	187
D (h)	0	0	0	0	147	0	0	0	113

## DISCUSSION

In an effort to determine the mechanisms of protective antioxidant activity during experimental Chagas' disease, the profiles of cytokines (especially of IFN- $\gamma$  and IL-10) were evaluated at the protein and mRNA level in mice infected with *T. cruzi* after dietary supplementation with vitamin E and Se. The results of the study show that vitamin E and Se supplementation results in the enhancement of IFN- $\gamma$  and suppression in the production of IL-10 compared with non-antioxidant treated mice. In this way, the antioxidants function to shift the T-helper (Th) 1/Th2 cytokine balance towards a Th1 response. These changes were observed at both the protein level and the transcriptional level and showed organ differences.

Cytokines play a fundamental role in the control of the immune response during chronic human Chagas' disease as well as other infectious diseases (Reis et al., 1997). Many experiments have been performed in an attempt to determine how the mammalian host develops resistance to *T. cruzi*. The results of many studies that have focused on cytokine regulation have shown that IL-12 is a key cytokine which powerfully induces NK cells to produce high levels of IFN- $\gamma$  (Cardoni et al., 1999). IFN- $\gamma$  then activates macrophages to produce NO, TNF- $\beta$ , and other factors which are capable of directly killing the parasite (Gazzinelli et al., 1992; Vespa et al., 1994; Holscher et al., 1998). During experimentally induced Chagas' disease, a Th1 immune response characterized

by IFN- $\gamma$  production and activation of macrophages plays a major role in resistance during acute *T. cruzi*-infection (Hoft et al., 1999). The balance of type 1 and type 2 cytokines then shifts in favor of IL-4 and IL-10 at 60 days or later following infection (Talvani et al., 2000). Endogenous IL-10 can down regulate an over-productive pro-inflammatory cytokine response preventing TNF- $\alpha$ -mediated toxic shock (Holscher et al., 2000). The balance between protective (e.g., IFN- $\gamma$ ) and exacerbative cytokines (e.g., IL-4 or IL-10) early in infection could be responsible for disease control and/or progression (Meyer et al., 1998).

Cytokine changes following *T. cruzi*-infection were confirmed by the results of this study. Compared to non-infected mice, IFN- $\gamma$  and IL-10 protein levels were increased in serum (Figure 2 and Figure 4) and mRNA levels were increased in the heart (Table 1a and Table 1b) of infected mice. IL-4 and IL-6 mRNA levels also increased in infected mice.

Compared with the non-antioxidant-treated but *T. cruzi*-infected groups, antioxidant-treated and *T. cruzi*-infected mice showed higher IFN- $\gamma$  levels, these differences of IFN- $\gamma$  levels between two groups were statistically significant ( $p < 0.05$ ) (Figure 2). In the groups of mice that were not infected by *T. cruzi*, very low IFN- $\gamma$  levels were observed in both experiment 1# and 2#. Even though the mean IFN- $\gamma$  levels appeared to be different, those differences between groups were not statistically significant. In mice treated for a longer time (8~9weeks) with antioxidants, IFN- $\gamma$  levels were higher than in mice treated for a shorter period of time (4~5weeks). The explanation for this result is complicated because antioxidant, age, and other factors would all be expected to be involved in this process. Based upon these results, antioxidant



supplementation with vitamin E and Se leads to an enhancement in serum levels of IFN- $\gamma$  in mice infected by *T. cruzi*.

In contrast, antioxidant supplementation resulted in a decrease in IL-10 levels in non-infected mice (Figure 4). These differences were statistically significant ( $p < 0.05$ ). These results also were consistent in both experiment #1 (mice treated 8-9 weeks) and experiment 2# (mice treated 4-5 weeks). Although there were observed differences in IL-10 levels in supplemented mice infected with *T. cruzi*, these differences were not statistically significant ( $p > 0.05$ ).

IFN- $\gamma$  and IL-10 are major elements of the Th1 and Th2 cytokine profiles, respectively. Based upon the above results, it appears that antioxidant supplementation shifts the cytokine response to Th1 in two ways: in *T. cruzi*-infected mice, the Th1/2 balance is shifted by increasing IFN- $\gamma$  levels, in non-infected mice, the Th1/2 balance is changed by decreasing IL-10 levels. Although there appeared to be other effects of diet on cytokine production, only the two changes mentioned above were statistically significant. ELISA analysis of cytokine production at the protein level demonstrated that IFN- $\gamma$  levels were higher in serum from *T. cruzi*-infected mice supplemented with vitamin E and Se as compared to non-antioxidant treated mice (Figure 2). These results were confirmed by RPA analysis of cytokine mRNA levels within the heart (Table 1).

Generally, the optimum vitamin supplementation level is considered to be the quantity that achieves the best growth rate, feed utilization, health (including immune competency), and provides adequate body reserves (McDowell, 2000). Vitamin E and Se both function as antioxidants. Both nutrients can impact the immune system and provide protection against a variety of diseases in humans and other animals (Sheffy and Schultz,

1979; Wise and Tomasso, 1993; Deshpande et al., 1996). When administered together, they show a synergistic effect in depressing lipid peroxide formation (Douillet et al., 1998). In addition, Vitamin E and Se show a positive synergistic effect on the immune system when supplemented in quantities in excess of recommended levels (Scheffy and Schultz, 1979). Combined supplementation of Se and vitamin E results in enhanced macrophage function (Wise and Tomasso, 1993) and also enhanced antibody production, cell proliferation, cytokine production, prostaglandin metabolism, and neutrophil function (Deshpande et al., 1996). In our laboratory, the combination of 400IU/kg vitamin E and 8ppm selenium was shown to provide the best overall protective effect in a murine model of experimental Chagas' disease (Hou, 1997). Based upon the results of the present study, the impact of these antioxidants on the production of immunoregulatory molecules such as cytokines is an important mechanism for their immunostimulatory effects.

Molecular oxygen and reactive oxygen intermediates (ROI) are required for host immunity to kill a variety of pathogens. Infection with *T. cruzi* stimulates tissues to increase ROI production which results in reduced tissue antioxidant levels in the host. Because the oxidant /antioxidant balance plays an important role in the regulation of cytokines, high concentrations of vitamin E and Se can neutralize the effects of excess reactive oxygen intermediates (Eugui et al., 1994). Depending upon the relative levels of oxidants /antioxidants, Th1 cytokines such as IFN- $\gamma$  may show increased production, whereas Th2 cytokines such as IL-10 and IL-4 levels may be decreased. During Th1-dominant immune responses, more active oxygen intermediates are produced and anti-microbial activity is enhanced. This response is important in the control and spread of the pathogen (Han and Meydani, 2000). Under these circumstances the antioxidants vitamin

E and Se may function to inhibit excessive inflammatory reactions. Therefore, the host immune response produces a protective function with limited pathological effects.

The work of Vilcek (1998) provided significant insight into the function of signal transduction pathways in the regulation of cytokine production. Redox status can regulate at least two transcription factors, nuclear factor (NF)- $\kappa$ B and activator protein (AP)-1. Many cytokine genes have been shown to have NF- $\kappa$ B and AP-1-binding sites in their promoter and enhancer regions. DNA binding and transactivation activities in transcriptional activator-dependent and cell type-dependent events can be up- or down-regulated by the relative levels of reduction /oxidation products (Sun et al, 1996). Vitamin E can inhibit NF- $\kappa$ B activation and this regulation has been shown to be cell type-specific (Sun and Oberley, 1996).

A second possible mechanism is the regulation of cytokine production by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). PGE<sub>2</sub> can affect Th1/Th2 cytokine secretion through IL-12 (Gately et al., 1998), and can up-regulate IL-6 production in macrophages (Thivierge and Rola-Pleszczynski, 1994). PGE<sub>2</sub> also has been shown to inhibit the early stages of T cell activation by decreasing IL-2 levels and down-regulating IL-2 receptor expression (Vercammen and Ceuppens, 1987). Vitamin E has been shown to decrease the production PGE<sub>2</sub> in old mice (Wu et al., 1998). Therefore, during vitamin E supplementation, IL-12 and IL-6 levels decrease whereas IL-2 levels increase and result in T cell activation.

In addition, at the cellular level vitamin E can help to protect the integrity of the cell membrane against ROI attack (Dutta-Roy et al., 1994; Deshpande et al., 1996). A stable cell membrane is necessary for normal cell function and as well as defense against invading pathogens.

The simultaneous presence of antigenic and antibody stimulation in the host during the chronic phase of Chagas' disease could explain the existence of both inflammatory and anti-inflammatory cellular reactivity detected in most patients (Dutra et al., 1997). The heart continues to be a major site of infection for the parasite, even after the acute phase of Chagas' disease (Tarleton & Zhang, 1999). Therefore, it is of interest to compare levels of inflammation between heart and spleen tissues in the antioxidant-supplemented mice and non-supplemented mice during experimental Chagas' disease.

One of the hallmarks of *T. cruzi*-infection is that the parasite demonstrates a preference for particular host organs or tissues. Furthermore, the parasite infection persists within tissues during the chronic stage of infection, which promotes high levels of oxidative processes, and causes severe destruction to the tissue. *Trypanosoma cruzi* stimulates a Th1 response within tissues which promotes the synthesis of increased levels of oxidative intermediates (Bussing et al., 1999). The results are chronic inflammation and pathologic damage to the tissue. As the level of oxygen intermediates varies in different organs (depending on the level of infection), antioxidants may affect the organs in different ways when the host is supplemented. The reduced numbers of blood-form trypomastigotes observed in mice in the present study were correlated with lower levels of inflammation, tissue parasitism, and tissue destruction. There is much evidence to suggest that CD8<sup>+</sup> lymphocytes are the main effector cells responsible for cardiac tissue destruction during *T. cruzi* infection (Brener & Gazzinelli, 1997). Nitric oxide generated from cardiac inducible nitric oxide synthase (NOS<sub>2</sub>) may be involved the pathogenesis of murine chagasic heart disease (Huang et al., 1999). In addition to mechanical damage by *T. cruzi*, substantial pro-inflammatory cytokine production within the myocardium is also

likely to participate in the pathophysiology of acute Chagasic cardiomyopathy (Chandrasekar et al., 1998). The infection-associated expression of cytokines and NOS<sub>2</sub> in the heart is clearly correlated with the severity of myocarditis (Huang et al, 1999). Chronic inflammation in Chagasic hearts is highly active and associated with a stable immunological pattern extending from the early acute stage of the infection through the late chronic stage (Zhang and Tarleton, 1999).

The results of the present study suggest that cytokine expression differs in different organs. The RPA analysis of the expression of cardiac cytokines demonstrated a significant increase in IL-6 and IL-10. IL-10 levels in heart tissue were higher than levels observed in the spleen. Similarly, IL-6 levels were elevated in the heart whereas the spleen showed no detectible levels of IL-6. IL-6 is secreted by many different kinds of cells and plays an important role in the regulation of immune and acute-phase responses. IL-6 is important in T cell activation, growth, and differentiation as well as B cell differentiation (Goldsby et al., 2000). IL-6 also is involved in the pathogenesis of many autoimmune disorders and in chronic proliferative disease (Hirano, 1994). In contrast to the results observed for IL-6, IL-2 and IL-4 were expressed at slightly higher levels in spleen cells than in heart tissue. IL-2 is a Th1 cytokine, which is essential in the immune response and required for the rapid proliferation of T cells. However IL-2 is inhibited in chronic Chagas' disease (Robert & Janovy, 1996). IL-4 is regarded as a Th2 cytokine and can stimulate B cells to make IgG1 and IgE. In a study by Zhang & Tarleton (1996), IL-6 was consistently observed in heart lesions appearing during the acute infection and persisting throughout the chronic stage of infection. In contrast, cells producing IL-2, IL-4, and IL-5 were not observed in the hearts of mice at any point during the infection. The authors also reported that the patterns of cytokine production in the heart were very different than profiles observed in lymphoid organs. Based upon the results of the their study, Zhang and Tarleton (1996) concluded that both inflammatory and anti-

inflammatory cytokines determine the pattern of the cellular response and the severity of disease in *T. cruzi* infection. Powell (1998) also showed that the differences in the profiles of cytokine mRNA may be related to the differential degree of cardiac pathology that develops in different strains of mice upon infection with *T. cruzi*. The results of the present study confirm that cytokine levels such as IL-6 and IL-10 are elevated in the heart as compared to the levels expressed in the spleen, indicating that Th2 responses may dominate and likely contribute to the pathology of Chagasic cardiomyopathy.

There are substantial data that support a possible role for autoimmunity in the pathogenesis of Chagas' disease, in which anti-parasite immune responses have cross-reactivity with self-molecules in *T. cruzi*-infected hosts (Kalil and Cunha-Neto, 1996). However, recent studies both in mice and humans indicate a positive association between tissue parasitism, inflammation, and severity of pathology induced by *T. cruzi* (Zhang & Tarleton, 1999). Strong evidence has been provided that parasite persistence within tissues is a primary cause of chronic Chagas' disease (Zhang & Tarleton, 1999). Many lines of evidences have shown that the persistence of *T. cruzi* at specific sites in the infected host results in chronic inflammatory reactivity and accounts for pathogenesis in chronic *T. cruzi* infections. It has become increasingly apparent that chronic Chagasic cardiac disease (CCCD) should be investigated and treated as a parasitic infection and not as an autoimmune disease (Tarleton, 2001).

In conclusion, the results of the present study have confirmed that antioxidants have a beneficial effect during experimental Chagas' disease. Furthermore, antioxidant supplementation with vitamin E and Se can tip the Th1/Th2 cytokine balance to a Th1-predominant response. This shift to a Th1 response was shown to be affected by infection status. The observed changes in cytokine synthesis showed organ differences between the

heart and spleen. Alterations in cytokine production within the heart may contribute to Chagas' cardiac pathology.

Much remains to be learned as regards how T cells may contribute to immune resistance as well as to the pathogenesis of *T. cruzi*-infection and chronic Chagasic cardiac disease. Further studies are needed to fully define the impact of antioxidants on the dynamics of cytokine regulation and the balance between protective immunity and immunopathology during experimental and human Chagas' disease.

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